Slc7a7 disruption causes fetal growth retardation by downregulating Igf1 in the mouse model of lysinuric protein intolerance

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Sperandeo MP, Annunziata P, Bozzato A, Piccolo P, Maiuri L, D’Armiento M, Ballabio A, Corso G, Andria G, Borsani G, Sebastio G. Slc7a7 disruption causes fetal growth retardation by downregulating Igf1 in the mouse model of lysinuric protein intolerance. Am J Physiol Cell Physiol 293: C191–C198, 2007. First published March 21, 2007; doi:10.1152/ajpcell.00583.2006.—The solute carrier family 7a member 7 gene (Slc7a7) encodes the light chain of the heterodimeric carrier responsible for cationic amino acid (CAA) transport across the basolateral membranes of epithelial cells in intestine and kidney. Mutations affecting Slc7a7 cause lysinuric protein intolerance (LPI), a multorgan disorder with clinical symptoms that include visceromegaly, growth retardation, osteoporosis, hyperammonemia, and hyperdibasicaminoaciduria. Here, we describe the consequences of inactivating Slc7a7 in a mouse model of LPI. The Slc7a7 mutation was generated by high-throughput retroviral gene-trapping in embryonic stem cells. The Slc7a7+/− mouse displayed intruterine growth restriction (IUGR), commonly leading to neonatal lethality. After heavy protein ingestion, the surviving adult animals presented metabolic derangement consistent with that observed in human LPI. IUGR was investigated by examining the expression of main factors controlling fetal growth. Insulin-like growth factor 1, the dominant fetal growth regulator in late gestation, was markedly downregulated as demonstrated by quantitative real-time RT-PCR, immunostaining and Western blot analysis in fetal liver. To further explore the pathophysiology of LPI, gene expression profiling analyses were carried out by DNA microarray technology in intestine and liver of adult Slc7a7+/− mice. Significant upregulation or downregulation (twofold or greater) was observed for 488 transcripts in intestine, and for 521 transcripts in the liver. The largest category of differentially expressed genes corresponds to those involved in transport according to Gene Ontology classification. This mouse model offers new insights into the pathophysiology of LPI and into mechanisms linking CAA metabolic pathways and growth control.

cationic amino acid transport; hyperdibasicaminoaciduria; neonatal lethality

LYSINURIC PROTEIN INTOLERANCE (LPI; OMIM no. 222700) is an autosomal recessive aminoaciduria caused by defective transport of cationic amino acids (arginine, lysine, ornithine; CAA) at the basolateral membrane of epithelial cells in the intestine and kidney. As a consequence, intestinal absorption and renal reabsorption of CAA are impaired with net loss of these amino acids. Clinical symptoms of LPI are variable and most frequently include vomiting, diarrhea, failure to thrive, growth retardation, hyperammonemia, osteoporosis, episodes of coma, lung involvement (mainly as alveolar proteinosis), and chronic renal disease (20). Metabolic derangement in LPI is linked to impaired urea cycle function due to lower plasma levels of arginine and ornithine. This leads to hyperammonemia after protein-rich meals and increased orotic aciduria. No pathogenetic mechanisms are known for many of the main clinical findings such as hepatoplenomegaly, alveolar proteinosis, renal involvement and stunted growth. In addition, these clinical symptoms respond poorly to the common treatment of LPI, which consists of dietary restriction of proteins and citrulline supplementation.

LPI is caused by mutations in the solute carrier family 7a member 7 gene (Slc7a7) which encodes the y+LAT-1 protein. y+LAT-1 is linked by a disulfide bond to solute carrier family 3 member 2 (Slc3a2, previously named 4F2hc) and together they comprise an amino acid transporter belonging to the heterodimeric amino acid transporter (HAT) family (3, 15, 22, 23, 27). This transporter, located at the basolateral membrane of epithelial cells, induces system y+L activity, which mediates CAA transport (15). Recently, functional studies of mutant solute carrier family gene alleles have suggested that the HAT defective in LPI may have a multiheteromeric rather than heterodimeric structure (22).

The mouse Slc7a7 gene maps on chromosome 14 (syntenic region of human chromosome 14) and its predicted amino acid sequence is highly homologous to the human protein (90.4% identity; 98.6% similarity). The murine y+LAT-1 induces system y+L activity and can form disulfide-linked heterodimers with human 4F2hc (16).

To elucidate the complex and mostly unknown pathophysiology of LPI, we attempted to recapitulate LPI in a mouse model. In collaboration with Lexicon Genetics (The Woodlands, TX), a Slc7a7 mutant clone was recovered by screening of the embryonic stem (ES) cell library generated by retroviral gene trapping and used to produce a Slc7a7-deficient mouse. In the present report we describe the analysis of phenotype of the Slc7a7-deficient mouse.

MATERIALS AND METHODS

Gene trapping and generation of the Slc7a7+/− mouse. The Slc7a7 gene was mutated using a gene trapping method by insertion

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mutagenesis with a retrovirual vector in ES cells (31). The identification
of the trapped gene was carried out by screening the OmniBank
(a library of mutated ES cell clones from Lexicon Genetics; http://
onmibank.lexgen.com/blat_frame.htm) using the Slc7a7 cDNA as a query.

OmniBank ES cell clone OST41878 was used to generate the
Slc7a7–/– mouse. All mice used for the present study were
of C57BL/6J genetic background obtained by backcrossing to C57BL/6J
strain mice for at least 10 generations. Mice heterozygous for
the mutant allele were bred to produce Slc7a7–/– mice.

Mice were housed at 24°C on a fixed 12:12-h light-dark cycle and
had free access to water and rodent chow (Mucedola, Milan, Italy).
All procedures involving animals were conducted in conformity with
our Institutional Animal Care and Use Committee guidelines that are
in compliance with the standards outlined in the guide for the Care
and Use of Laboratory Animals.

Both Slc7a7–/– mice were fed a diet contained 8% protein (special
diet produced ad hoc by Mucedola) and citrulline supplementation
(100 mg/ml solution; administered at 100 μg/g of body wt/diet)
(Sigma-Aldrich, Italy).

Genotyping of Slc7a7 fetuses and mice colony. Genomic DNA
was extracted by standard protocols from tail biopsies isolated from
3-wk-old progeny of matings and from embryonic day (E) 16.5
fetuses. Fetuses and mice were genotyped by PCR using primers A
and B, located in intron 2 of the Slc7a7 gene and surrounding the
gene-trap insertion site, and primer LTRrev (a vector specific primer)
(Fig. 1A).

The sequences for primers A and B are 5′-GATGAAGT-
GATCCTAGCCGTTAG-3′ (for A) and 5′-GCAGCCTCTATGTCA-
CAGGGCG-3′ and LTRrev 5′-AAATGGCGTTACTTAAGCTAGCT-
TG-3′ (for B). DNA (100 ng) purified from mice and fetal tail genomic
was used as template for PCR in a 25 μl reaction volume. PCR conditions
were: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 39 cycles.
PCR-sexing of mouse fetuses was performed as described in Ref. 12.

Northern blot analysis of Slc7a7–/– mice. Total RNA was pre-
pared from kidneys using TRIzol reagent (Invitrogen) according to
the manufacturer’s instructions and processed for Northern blot analysis.
A cDNA fragment that spans the two Slc7a7 exons flanking the
retrovirus integration site was used as the hybridization probe.

Anatomical examination and insulin-like growth factor 1 immuno-
histochemistry. Detailed gross and histological examinations were
performed on whole mount fetuses and selected tissues such as
intestine, liver, spleen, lung, kidney, and brain isolated from fetuses
and adult mice. Whole mount fetuses and selected tissues of each
mouse (fetus and adult, respectively) were weighed and then fixed by
immersion in 4% paraformaldehyde for 24–48 h. After fixation, tissues
were routinely processed and embedded in paraffin. Briefly, 4-μm
thick sections were deparaaffinized in xylene and rehydrated and
then stained with hematoxylin and eosin for morphological assessment.
Fixed liver tissues isolated from fetuses were processed for immuno-
histochemistry, as described in Ref. 10. Polyclonal goat antiserum to
mouse insulin-like growth factor 1 (Igf1) were used for the staining.
(Santa Cruz Biotechnology, Santa Cruz, CA). The sections were
viewed with a Nikon microscope. Pictures were processed and assem-
bled using Adobe PhotoShop. To prevent observer bias, all histologi-
cal specimens were coded and examined without knowledge of
animal age and genotype.

Igf1 Western blot analysis. Liver tissue extracts were isolated from
fetuses and processed in lysis buffer with protease inhibitor cocktail.
Ten micrograms of extracts were subjected to SDS-PAGE and trans-
ferred onto a polyvinylidine difluoride membrane. The membranes
were incubated with polyclonal goat antiserum to mouse Igf1 (Santa
Cruz Biotechnology).

Quantitative real-time RT-PCR. Real-time PCR was performed according to
the recommendations supplied by Applied Biosystems
(http://europe.appliedbiosystems.com/). All primers for real-time
PCR were purchased from Applied Biosystems as AssaysOnDemand.
All reactions were performed in triplicate. The abundance of the

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Fig. 1. Schematic representation of the strategy for targeted disruption of
solute carrier type 7a7 (Slc7a7). A: deficient (−/−) Slc7a7–/– mice were
generated from OmniBank embryonic stem (ES) cell clone OST41878, which
contains a gene-trap vector insertion in the second intron of the Slc7a7 gene.
A retroviral vector contains a splice acceptor sequence (SA), followed by
promoterless selectable β-Geo, a functional fusion between the β-galactosi-
dase and neomycin resistance genes, with a polyadenylation signal (pA).
Insertion of the retroviral vector into Slc7a7 intron 2 leads to the splicing of
the Slc7a7 upstream exons into this cassette generating a fusion transcript.
The vector contains a promoter that is active in ES cells (Pgk1; phosphoglycerate kinase-1
promoter), followed by the first exon of the Bruton’s tyrosine kinase (Btk) gene
upstream of the splice donor (SD) signal. Splicing from this signal to the exons
downstream of the insertion gives rise to a fusion transcript that contains
termination codons in all reading frame to prevent translation of downstream
fusion transcript. B: PCR genotyping of wild-type (+/+), heterozygous (+/−), and
Slc7a7–/– mice. Primers A and B flank the gene-trap insertion in intron 2
of the Slc7a7 gene and amplify a PCR product representing the wild-type
(+/+) allele. The mutant allele was detected in heterozygous (+/−) and
Slc7a7–/– mice with primer B and a vector-specific primer, LTRrev, comple-
mentary to the gene-trapping vector. C: Northern blot analysis of Slc7a7
expression in kidney total cellular RNA from wild-type (+/+), heterozygous
(+/−), and Slc7a7–/– fetuses. The probe is a Slc7a7 partial cDNA that spans
the two exons flanking the retrovirus integration site. The same blot was
reprobed with β-actin as a loading control.

target mRNAs was calculated relative to a reference mRNA (β2
microglobulin). Relative expression ratios were calculated as
R = 2[Ct (β2 microglobulin) − Ct (test)], where R is ratio, Ct is the cycle
number at the threshold, and test refers to the tested mRNA. The confidence
interval was fixed at 95%.

Urinary collection and analysis of amino acids and orotic acid in
urine samples. Twenty-four-hour urine samples were obtained by
placing the mice in specially designed minimechanical cages. Quanti-
tation of amino acids was performed by liquid chromatography/mass
spectrometry (LC/MS). Orotic acid analysis was carried out by gas
cromatography/mass spectrometry (GC/MS).

Determination of ammonia concentration. Blood was taken from
tails of Slc7a7–/– mice and wild-type controls. Blood ammonia levels
were determined using the Ammonia Checker II system (Arkay),
after dilution of blood samples 1:4 in water. Measurement range of
the Ammonia Checker II system is 10–400N-μmol/dl (7–286 μmol/l).

Array hybridization, scanning, and data analysis. Liver and intes-
tine were harvested from two Slc7a7–/− adult mice and two littermate
controls of matching ages kept on the same diet as the mutant animals.
RNAS were pooled according to the genotype. The array studies were

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RESULTS

Generation of Slc7a7−/− mice. We screened the OmniBank library (Lexicon Genetics) and identified an ES cell clone containing a gene-trap insertion in Slc7a7 (clone OST41878), which was used to generate the Slc7a7−/− mouse. Analysis of the 500-bp sequence surrounding the gene-trap insertion site showed that the gene-trap vector is located in intron 2 of Slc7a7 downstream of the ATG-containing exon (Fig. 1A). The heterologous exon 1 of the Bruton tyrosine kinase (Btk) gene in the gene-trap cassette is a noncoding exon that contains in-frame stop codons that eliminate the likelihood of expression of a protein from the trapped Slc7a7 locus (31). A genotyping strategy was designed using primers that flank the genomic insertion site of the gene-trap vector and a primer (LTRrev) specific for the gene-trap vector (Fig. 1B). Slc7a7 targeting resulted in a null allele as demonstrated by the absence of Slc7a7 mRNA by Northern blot analysis in Slc7a7−/− fetuses (Fig. 1C) and by quantitative real-time RT-PCR and DNA microarray in adult Slc7a7−/− mice.

Genotyping of 606 pups generated in >200 Slc7a7+/+ intercrosses revealed ratios of genotypes (28.4% wild-type, 68.6% heterozygotes, 3% Slc7a7−/− homozygotes) inconsistent with Mendelian segregation of a recessive trait. This indicated either the in utero demise of Slc7a7−/− fetuses or the death of Slc7a7−/− newborn animal shortly after birth. No difference of sex distribution was found among heterozygous mice; heterozygotes were healthy, fertile, and identical to wild-type littermates. Only 2 Slc7a7−/− mice survived, whereas 16 of them died within 24 h of birth. Eventually, the high rate of lethality allowed us to reach an almost pure C57BL/6J genetic background by backcrossing heterozygous offspring before the birth of the two surviving Slc7a7−/− mice.

The Slc7a7 lethality was further defined by genotyping fetuses at E16.5. At this developmental stage, no significant differences were found for segregation and sex ratios among the three genotypes. This confirmed the suspicion of maternal cannibalism of Slc7a7−/− pups soon after delivery. Fostering

soon after birth was tried without success. Dietary supplementation of pregnant dams with either citrulline or arginine did not increase the survival rate of Slc7a7−/− animals at birth.

At E16.5, Slc7a7−/− fetuses appeared smaller than controls by gross examination (Fig. 2A) and presented a developmental delay of 2–3 days as observed by histological examination (9). At birth, Slc7a7−/− pups were smaller and appeared less vital than their littermates (Fig. 2B).

Both surviving Slc7a7−/− mice were fed a low-protein diet with citrulline supplementation after weaning. They showed lasting growth retardation compared with wild-type siblings kept on the same diet and citrulline supplementation. The older animal, a male, survived 25 mo without showing signs of disease apart from stunted growth (Fig. 2C). At 25 mo, a normal diet was introduced and the citrulline supplementation was withdrawn. The mouse rapidly presented progressive hypotonia, tremors, weight loss (~63% of the initial weight), and died 15 days later. The second animal, a female, had several unsuccessful matings followed by a single pregnancy that ended prematurely on the day 18. All of the pups were cannibalized by the mother as evidenced by remnants of four pups. The dam displayed acute neurological symptoms (tremors, severe hypotonia, and nonresponsiveness to external stimuli) 3 h after delivery, and died shortly thereafter.

Detailed gross and histological studies were performed, as described in MATERIALS AND METHODS. No significant differences were found between Slc7a7−/− and wild-type animals apart from bone developmental delay and spleen enlargement (weights of spleens: 240 mg, Slc7a7−/− male mouse; 220 mg, Slc7a7−/− female mouse; 102 mg ± 5, controls; P < 0.003).
Biochemical investigations confirm metabolic derangement of LPI in adult Slc7a7−/− mice. The biochemical hallmarks of LPI are increased renal excretion of CAA and hyperammonemia after oral protein load. Therefore, we measured CAA urinary levels in both Slc7a7−/− animals when fed a restricted diet with citrulline supplementation and, in the male mouse, when it was put on a normal diet at 25 mo. The rapid and severe clinical course of the female Slc7a7−/− animal prevented collection of urine samples. Both Slc7a7−/− adult mice showed elevated urinary excretion of arginine, ornithine, and orotic acid when kept on a low-protein diet (Table 1). Lysine excretion strongly increased when the Slc7a7−/− male mouse was put on a normal diet without citrulline supplementation (Table 1). CAA and orotic acid renal excretions were not significantly different between wild-type and heterozygous mice. Hyperammonemia was found in both Slc7a7−/− animals in blood samples taken soon after death (mean ammonia concentration ± SE in μmol/l: 135.5 ± 40.8 for Slc7a7+/++; 137.3 ± 24.7 for Slc7a7+/−, 1,050 and 1,530 for the male and the female Slc7a7−/− mice, respectively).

To exclude the possibility that the embryonic growth retardation of Slc7a7−/− mice was due to lower CAA availability we measured CAA levels in amniotic fluids at E16.5. Amniotic fluids were collected and pooled after genotyping of fetuses. No differences in CAA level were observed among the pooled samples representing the three different genotypes.

Intrauterine growth restriction of fetal Slc7a7−/− mice. All of the Slc7a7−/− mice showed intrauterine growth restriction (IUGR). In addition to the determination of CAA levels in amniotic fluids, the expression of genes involved in CAA transport (Slc7a1, Slc7a2, Slc7a6, and Slc3a2) was investigated in placentas of E16.5 fetuses by quantitative real-time RT-PCR. No significant differences were observed between Slc7a7−/− animals and controls. Downregulation of Slc7a2 (0.74-fold) was found in liver of Slc7a7−/− fetuses by quantitative real-time RT-PCR.

To elucidate the pathogenesis of severe IUGR we examined the fetal liver expression of genes encoding the insulin-like growth factors 1 and 2 (Igf1 and Igf2), and their binding proteins (Igfbp1-6). Igf1 and Igf2 mRNA levels were, respectively, 3.2 and 1.7-fold lower in Slc7a7−/− animals than in wild-type animals (Fig. 3A). In the Slc7a7−/− fetus that displayed the smallest body size, liver Igf1 and Igf2 were reduced by 7.4- and 4.0-fold, respectively (Fig. 3B). These results differ from those in the adult Slc7a7−/− animals, in which Igf1 and Igf2 expression levels were unaltered relative to controls (in liver the value of Igf1 was 0.968, whereas the value of Igf2 was 0.8925). Composite results were found for Igfbps in Slc7a7−/− fetuses: downregulation of Igfb1 (2.6-fold), upregulation of Igfbp2 (3.6-fold) and Igfbp6 (3.2-fold) (Fig. 4A). In the smallest Slc7a7−/− fetus, Igfbp1 and Igfbp4 were downregulated by 48- and by 8.3-fold, respectively. The same animal showed upregulation of Igfbp2, Igfbp5, and Igfbp6 by 7.4-, 3.5-, and 31.6-fold, respectively (Fig. 4B).

Expression of Igf1 was investigated at the protein level by immunostaining and Western blot analysis of liver samples. The immunohistochemical labeling of Igf1 showed evenly reduced signals from Slc7a7−/− fetuses compared with wild-type controls (Fig. 5A). In the Western blot, Igf1 cross-reacting material of Slc7a7−/− fetuses was reduced to 2 to 65% of the wild-type controls (Fig. 5B).

Table 1. Metabolic derangement in Slc7a7−/− mice

<table>
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<tr>
<th>Slc7a7−/−</th>
<th>Ornithine</th>
<th>Lysine</th>
<th>Arginine</th>
<th>Orotic Acid</th>
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<tr>
<td>Male Slc7a7−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Low-protein diet</td>
<td>108.5</td>
<td>52.36</td>
<td>109.4</td>
<td>151.7</td>
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<tr>
<td>+ Normal diet</td>
<td>134.31</td>
<td>165.72</td>
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<tr>
<td>Slc7a7−/− (n = 3)</td>
<td>333.7</td>
<td>79.7</td>
<td>471.1</td>
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<tr>
<td>Wild type (n = 3)</td>
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<td>23.7 ± 6.5</td>
<td>2.7 ± 1.2</td>
<td>66.3 ± 12.7</td>
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<tr>
<td>Slc7a7−/− (n = 3)</td>
<td>14.4 ± 0.6</td>
<td>67.3 ± 16.61</td>
<td>9.31 ± 3.2</td>
<td>57.2 ± 2.2</td>
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Values are means ± SE. Acids listed in header refer to urinary amino acid and orotic acid levels were determined by liquid chromatography mass spectrometry analysis and are given as nmol/mMol of creatinine.
expression profiles of Slc7a7−/− and control mice by using DNA microarrays and quantitative real-time RT-PCR.

Microarray analysis was carried out on small intestine and liver harvested from the two adult Slc7a7−/− mice and from two normal littermate controls kept on the same diet and citrulline supplementation. Pooling of RNAs was performed to reduce the variability that may occur with single sample, and to obtain sufficient amount RNA for DNA array hybridizations.

To facilitate the one-by-one analysis of differentially expressed genes, we used the Expression Analysis Systematic Explorer software to automate the process of creation of descriptive annotation tables. Furthermore, to translate the data into a more meaningful biological context and to highlight sets of functionally related genes, the differentially expressed datasets were subsequently organized into GO groupings using the OE software. Table 2 shows the results of the OE analysis of differentially expressed genes relative to the GO biological process categories. These combined analysis allowed us to pinpoint among the differentially expressed genes the ones that appear relevant to the molecular and clinical features of LPI and to the phenotype observed in Slc7a7−/− mice.

In the small intestine of Slc7a7−/− mice, greater than twofold upregulation or downregulation was observed for 154 and 334 transcripts, respectively. The solute carrier family 34 Pi cotransporter involved in small intestinal absorption of P i. In the liver samples, 281 genes were upregulated and 240 genes were downregulated. Igfbp1 was the gene with the greatest upregulation (71.1-fold) in Slc7a7−/− mice. Microarray data relative to Slc34a2 and Igfbp1 were confirmed by quantitative real-time RT-PCR. The complete expression data-

Table 2. Onto-Express analysis of genes differentially expressed between Slc7a7 null and control mice in liver and intestine

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Biological Process refers to only the five most statistically relevant Gene-Ontology biological processes are shown for each dataset.
sets were submitted to GEO database (http://www.ncbi.nlm.nih.gov/geo/).

In the next two sections, we report the results (confirmed by quantitative real-time RT-PCR) concerning transcripts, identified in the entire dataset of differentially expressed genes, that may have functional implications for the pathophysiology of LPI.

Dysregulation of urea cycle and arginine metabolism in adult Slc7a7−/− mice. Liver microarray expression profiles of genes involved in the urea cycle revealed upregulation of arginase type I (EC 3.5.3.1), argininosuccinate lyase (EC 4.3.2.1) and glutamate dehydrogenase 1 (EC 1.4.1.3) (3.0-, 3.0-, and 2.1-fold, respectively) and 2.3-fold downregulation of ornithine transcarbamylase (EC 2.1.3.3). In intestine, the isoform II of arginase (EC 3.5.3.1) was upregulated by 5.1-fold. In contrast to isoform I, arginase type II has a mitochondrial localization and transforms arginine into ornithine and urea in an alternative metabolic pathway.

CAA transporter genes differentially expressed in adult Slc7a7−/− mice. GO analysis revealed that the most differentially expressed genes in the intestine and liver are in the biological process category termed “transport”.

Within the SLC7 family, the solute carrier family 7 member 2 gene (Slc7a2) showed a 4.3-fold upregulation in liver. However, the solute carrier family 7 member 9 gene (Slc7a9), which is involved in the transport of cationic plus neutral amino acids (system b0,+), was 7.0-fold downregulated in intestine. The solute carrier family 7 member 6 gene (Slc7a6) is ubiquitously expressed and encodes y+LAT-2, the light chain of the second transporter with y+L activity (26). The expression of Slc7a6 (not present on the DNA microarray chip) was tested by quantitative real-time RT-PCR and was found to be upregulated by 5.8 fold in liver from Slc7a7−/− mice.

DISCUSSION

The molecular bases underlying the multiorgan involvement of LPI are still unclear. Some of the biochemical LPI findings such as hyperammonemia and increased ornotic acid production might be explained by an intracellular CAA depletion in hepatocytes. However, it is much more difficult to understand mechanisms underlying important clinical features such as visceromegaly, postnatal growth retardation, early osteoporosis, or the involvement of the lung or kidney. An animal model may provide crucial insights into the pathophysiology of this disorder and also allow testing of innovative treatments.

In collaboration with Lexicon Genetics, we generated a genetically modified mouse in which Slc7a7 gene function was disrupted. Growth retardation was an ongoing feature of the two surviving Slc7a7−/− mice compared with normal littermates kept on the same diet and with citrullinuria supplementation.

An acute metabolic derangement, identical to that found in human LPI, was observed after a planned withdrawal of the special diet for the male mouse, and after an accidental protein overload for the female. This metabolic derangement had a fast onset and rapidly caused the death of both animals after severe hyperammonemic neurological symptoms. Other biochemical characteristics of human LPI, such as massive urinary excrections of arginine, ornithine, and ornotic acid, were present even when both animals were kept on the special dietary regimen. Hyperlysinsuria appeared only in the acute stage of the disease as seen in urine collected from the Slc7a7−/− male animal. The transient normal lysine excretion can be explained by the low protein content of the diet, similar to what is observed in human patients. In fact, excretion of lysine (as well as ornithine and arginine) may be within normal values when LPI patients spontaneously restrict their protein intake before the diagnosis is established (20). Gross and histological studies did not reveal organ-specific pathology apart from splenomegaly.

The neonatal lethality of the present LPI mouse model is in contrast to the natural history of human LPI where death may occur as a consequence of either an acute metabolic derangement or a severe complication such as pulmonary alveolar proteinosis. The lethality of the murine model is due to IUGR which is not present in human LPI patients although it was reported in unaffected children born to LPI mothers (25). Determination of amniotic CAA levels in Slc7a7−/− fetuses revealed values comparable to those found in normal controls thus ruling out CAA depletion as a primary cause of IUGR. In addition, no differences were found for placental expression profiles of genes involved in CAA transport (Slc7a1, Slc7a2, Slc7a6, and Slc3a2), with the exception of Slc7a7. To unravel the mechanism causing IUGR in Slc7a7−/− fetuses, we investigated the expression of the main genes controlling intracellular growth: insulin-like growth factors (Igfs) and their binding proteins (Igfbps) (5). The Igf1 transcript was downregulated 3.2 fold in livers pooled from Slc7a7−/− fetuses. The smallest Slc7a7−/− fetus displayed the lowest Igf1 expression (7.4-fold lower than controls).

In support of the quantitative real-time RT-PCR data, both immunohistochemical labeling and Western blot analysis showed marked reduction of Igf1 signals. Igf2 was significantly underexpressed only in the smallest Slc7a7−/− fetus. Igf1 plays a pivotal role in promoting intrauterine growth and, during this developmental stage, is independent from growth hormone control. Like Slc7a7−/− fetuses, Igf1-deficient mice exhibit a 40% decrease in birth weight and >95% of puppies die perinatally (11, 17). Therefore, marked underexpression of Igf1 in Slc7a7−/− mice is sufficient to explain the early lethality. A complex pattern of dysregulation emerged from expression profiling of the genes encoding Igf binding proteins. Igfbps comprise a family of six homologous proteins that bind Igfs and control their bioavailability. At one extreme we found downregulation of Igfbp1 and Igfbp4, and at the other a strong upregulation of Igfbp2 and Igfbp6. The degree of downregulation of Igfbp1, a binding protein specifically produced in liver, seems to correlate with that of the Igf1 transcript. In fact, both Igfbp1 and Igf1 reached the lowest levels of expression in the smallest Slc7a7−/− fetus. It is known that Igfbp1 has an inhibitory role on Igf-stimulated growth and differentiation. In addition, IUGR caused by placental insufficiency and in utero hypoxia is associated with elevated levels of Igfbp1 (28, 29). The underexpression of Igfbp1 in Slc7a7−/− fetuses may simply reflect the inverse relationship between this binding protein and Igf1 (14). Transgenic models overexpressing either Igfbp2 or Igfbp6 show reduced postnatal growth of the entire body, for the former, or of selected organs, for the latter (2, 8). In Slc7a7−/− mice, dysregulation of the Igf axis is not related to defective transport of CAA across placenta. Our data suggest that the downregulation of Igf1 in Slc7a7−/− mice has to be explained by an altered intracellular CAA homeostasis due to Slc7a7 ablation. Postnatal growth delay, a frequent

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clinical finding in human LPI, is not caused by the protein-restricted diet but it might be related to the alteration of the GH/IGF1 axis as recently reported (6).

Furthermore, arginine has been demonstrated to stimulate IGF1 production and collagen synthesis in osteoblast-like cells but the intimate mechanism of regulation remains unclear (4).

To further explore the diverse regulation of genes likely related to LPI pathophysiology, microarray-based gene expression profiles were obtained from the intestine and liver of the two adult Slc7a7+/− mice. The rationale underlying the choice of these organs was that Slc7a7 is normally expressed in the intestine and not in the liver, even though the liver is also involved in the pathogenesis of LPI.

Our study revealed that the absence of a functional Slc7a7 mRNA results in the dysregulation of >400 and 500 transcripts in intestine and liver, respectively. The largest enriched GO category of differentially expressed genes corresponds to those involved in transport. This is not surprising because intestine expresses a large number of transporters, molecules that play a direct role in the absorption of various compounds from the lumen. Similarly, several transporter families mediate uptake of chemicals into, and excretion of chemicals from, the liver.

In the liver, upregulation of Slc7a2 and Slc7a6 may reflect the need to restore the intracellular CAA pool since both genes are involved in CAA transport. Upregulation of Slc7a6 was found in a previous study carried out in lymphoblasts from LPI patients (19). Interestingly, Slc7a6 has the same transport activity as Slc7a7 and is ubiquitously expressed. However, its overexpression, as seen in the present animal model, does not seem to produce a compensatory effect on CAA transport. Slc7a6 probably plays a different role which is still unclear. Transcripts encoding enzymes involved in the urea cycle are all upregulated with the exception of ornithine transcarbamylase. This general upregulation may be interpreted as an attempt to compensate for the hyperammonemic condition. In fact, conditions causing increased ammonia production such as starvation or high protein intake lead to increase of all urea cycle enzymes by a transcriptional control (24). As to the downregulation of the ornithine transcarbamylase transcript, this gene responds to other stimuli (e.g., glucocorticoids) differently from all the other enzymes of the urea cycle. In contrast to what is observed in Slc7a7+/− fetuses, in the adult Slc7a7+/− animals Igfbp1 was upregulated by 71-fold, representing the most differentially expressed transcript in liver. High levels of Igfbp1 may be responsible for the severe growth inhibition and the bone developmental delay observed in these Slc7a7+/− mice despite normal levels of Igf1 expression. This dramatic increase of Igfbp1 might also have related to the protracted critical illness as suffered by the male Slc7a7+/− mouse (13).

In the intestine, the Slc7a9 and Slc34a2 genes both display marked downregulation. Slc7a9 encodes the light chain of b(0,+)AT, the transport system of cystine and CAA across the apical membrane of epithelial cells of intestine and kidney. The 7.0-fold downregulation of this transcript might be explained as a compensatory mechanism to reduce intracellular accumulation of CAA in intestinal epithelial cells by hampering their transport at the basolateral membrane.

Slc34a2, which encodes a Na+-Pi cotransporter responsible for intestinal phosphate absorption (30), shows a 54.8-fold downregulation. Mutations of SLC34A1, the Na+-phosphate cotransporter expressed in kidney, are associated with hypophosphatemia and osteoporosis in human subjects (18). Whether defective Slc34a2 transport activity might contribute to phosphate depletion is still unclear, although osteoporosis is an early complication commonly observed in LPI patients. The underexpression of Slc34a2 in intestine of Slc7a7+/− mice suggests new studies on mechanisms linking CAA and phosphate absorption.

The Slc7a7+/− mouse model described here offers new insights into the pathophysiology of LPI and into mechanisms linking CAA transport with prenatal growth control.

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REFERENCES


